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Title: A versatile, high-efficiency platform for CRISPR-based gene activation

Authors and Affiliation

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Abstract

CRISPR-mediated transcriptional activation (CRISPRa) is a powerful technology for inducing gene expression from endogenous loci with exciting applications in high throughput gain-of-function genomic screens and the engineering of cell-based models. However, current strategies for generating potent, stable, CRISPRa-competent cell-lines present limitations for the broad utility of this approach. Here, we provide a high-efficiency, self-selecting CRISPRa enrichment strategy, which combined with piggyBac transposon technology enables rapid production of CRISPRa-ready cell populations compatible with a variety of downstream assays. We complement this with a new, optimized guide RNA scaffold that significantly enhances CRISPRa functionality. Finally, we describe a novel, synthetic guide RNA tool set that enables transient, population-wide gene activation when used with the self-selecting CRISPRa system. Taken together, this versatile platform greatly enhances the potential for CRISPRa across a wide variety of cellular contexts.

Main

Recent advances in genome engineering technology have enabled unprecedented opportunities for exploring the consequences of altered gene function or expression in a variety of model systems.^{1,2} Driving many of these efforts has been the adaptation of the microbial CRISPR/Cas9 system for use in eukaryotic organisms³. Cas9's defining feature, as an easily programmable RNA-directed double stranded DNA (dsDNA) nuclease, has inspired the creation of genome-scale perturbation libraries and subsequent loss-of-function screens across hundreds of human cell lines⁴⁻⁶. These screens have proven invaluable for uncovering genotype and cell lineage-specific gene dependencies, which continue to inform basic as well as clinical research efforts⁷.

Cas9 can also be engineered for expanded use beyond the creation of targeted dsDNA breaks. The fusion of transcriptional repressor or activator domains to a nuclease-dead form of Cas9 (dCas9), enables CRISPR-mediated transcriptional interference (CRISPRi) or activation (CRISPRa), respectively⁸⁻¹⁰. CRISPRa is a compelling technology for the activation of endogenous gene expression in disease models or gain-of-function screens¹¹⁻¹³. A host of

46 activator domains and transgene expression systems have been engineered to enable the
47 production of CRISPRa-competent cells¹⁴. However, current strategies for engineering CRISPRa
48 transgenic cell-lines are inefficient, prone to silencing, and often necessitate a labor-intensive
49 single-cell cloning process. Gene and cell line-dependent variability pose further limits on the
50 scalability of CRISPRa.

51
52 Here, we provide a comprehensive platform based on the Synergistic Activation Mediator
53 (SAM)¹³ CRISPRa concept, that takes advantage of a self-selection mechanism to create uniform,
54 potent, and stable CRISPRa-competent cell populations without the need for clonal selection. In
55 addition, we demonstrate the effectiveness of a new SAM-compatible single-guide RNA (sgRNA)
56 variant that both improves the function of sub-optimal sgRNAs and enables activity from sgRNAs
57 found to be inactive with earlier-generation scaffolds. We show that this new sgRNA format is not
58 only capable of facilitating stable gene expression, but that it can also be used for transient target
59 activation through a novel, chemically-synthesized guide RNA tool set. Altogether, this new, user-
60 friendly platform maximizes the potential for CRISPRa across a breadth of cell-based contexts
61 and genetic loci.

62
63
64 **Results**

65
66 **A self-selecting CRISPRa strategy for the rapid generation of stable, high-efficiency**
67 **CRISPRa cell populations**
68

69 Several dCas9-activator concepts have been described¹². In pilot experiments, we observed
70 consistent evidence of target activation with the Synergistic Activation Mediator (SAM) system
71 (data not shown), and selected this platform for optimization studies. The SAM system poses a
72 challenge, however, owing to the size and number of discrete elements that must be introduced
73 in order to create a stable CRISPRa-ready cell population. These include a dCas9-VP64 fusion
74 protein, an MCP- (MS2 coat protein) p65-HSF1 co-activator fusion protein (MPH), and any
75 number of selection markers. Combined, these components and their associated regulatory
76 sequences exceed the conventional limit for efficient lentiviral packaging¹⁵, often necessitating a
77 multi-vector delivery strategy^{13,16}. The piggyBac transposon system¹⁷, on the other hand, allows
78 for both a higher cargo capacity and the incorporation of multiple transgene cassettes within a
79 single vector. PiggyBac-based strategies have been utilized for CRISPRa-based cell line
80 generation^{18,19}, but, similar to lentivirus, its use results in random genomic integration and
81 functional heterogeneity within the cell population. The resulting low efficiency populations are
82 often incompatible with demanding applications like functional genomic screening without the
83 further derivation and characterization of high efficiency clones. Due to the laborious and time-
84 consuming nature of this process we aimed to develop a simple and efficient bulk selection
85 method to enrich for stable, uniform, and potent CRISPRa-expressing cell populations.

86
87 To this end, we designed a series of multi-component CRISPRa piggyBac vectors which
88 employed individual selection strategies for the enrichment of transgenic cells (Fig. 1a). In each
89 context, expression of the CRISPRa activator elements was driven by a human EF1 α promoter,
90 and this was complemented by a distinct mechanism for the transcription of a co-expressed

91 puromycin resistance gene (*puro^r*). Similar to previous studies, we created a *dual promoter*
92 selection vector¹⁹ (Fig. 1a-top row) where *puro^r* was driven by an independent promoter (PGK)
93 and a *single transcript* vector²⁰ where *puro^r* was transcriptionally linked to the CRISPRa machinery
94 (Fig. 1a-middle row). The theoretical selection pressure exerted by these strategies should be on
95 maintaining transgene genomic integration in the case of the dual promoter vector and on
96 sustained transgene expression in the case of the single transcript system (Fig. 1a-right column).
97 As a readout for CRISPRa function we also incorporated a GFP reporter downstream of a self-
98 activating (SA) promoter, which could be activated only in the presence of functional CRISPRa
99 machinery and a co-expressed SA-targeting guide RNA. Building on the self-activating concept,
100 we devised a third strategy, which we term *CRISPRa selection* (CRISPRa-sel), where the *puro^r*
101 gene is driven by the self-activating promoter and linked to the GFP reporter (Fig. 1a-bottom row).
102 Unlike the dual promoter or single transcript approaches, in the CRISPRa-sel context there is an
103 absolute requirement for each cell to maintain functional CRISPRa in order to survive in the
104 presence of puromycin.

105
106 We evaluated the relative efficiency of each selection strategy in the human K562 cell line.
107 Following puromycin selection, the individual populations were infected with lentiviral vectors
108 expressing SAM-compatible sgRNAs targeting the promoter proximal regions of five cell surface
109 receptor genes (Extended Data Fig. 1a). Quantitative RT-PCR (qRT-PCR) data from each
110 condition revealed consistently-improved gene activation with the CRISPRa-sel system relative
111 to the other formats (Fig.1b). We further used flow cytometry to quantitatively assess cell surface
112 protein expression on an individual cell level (Fig.1c,d; Extended Data Fig. 1b). This analysis
113 further demonstrated a dramatic enhancement in gene activation with the CRISPRa-sel system
114 both in terms of absolute protein expression, by way of normalized median fluorescence intensity
115 (MFI), and percent positive-stained cells. While the dual promoter and single transcript systems
116 showed highly heterogenous populations with only a small number of active cells, the CRISPRa-
117 sel strategy resulted in a substantial improvement in the proportion of active cells, which in some
118 cases achieved near population-wide activation (i.e. PD-L1 and CD2). To confirm the broad
119 applicability of our findings, we expanded our analysis to two additional, unrelated human cell
120 lines (Extended Data Fig. 1 c,d) where similar trends were observed. Importantly, potent
121 endogenous gene activation with the CRISPRa-sel format suggested the self-activating circuit did
122 not interfere with gene expression induced by separate, lentivirally-delivered sgRNAs.

123
124 In addition to endogenous target activation, we evaluated whether our integrated
125 CRISPRa-dependent GFP reporter was effective at identifying CRISPRa-competent cells. To our
126 surprise, we found that GFP intensity did not reliably correlate with endogenous target gene
127 activation across the tested selection formats and cell lines (Extended Data Fig. 2). Although a
128 trend towards correlation was observed for the dual promoter format (Extended Data Fig. 2a-left),
129 there was high variability in the single transcript and CRISPRa-sel contexts (Extended Data Fig.
130 2a-center, right). To further evaluate the relationship between GFP expression and endogenous
131 gene activation in the CRISPRa-sel context, we expanded our analysis to a second endogenous
132 target gene (CD2) (Extended Data Fig. 2b) and observed similarly weak correlations. Despite
133 this observation when analyzed in bulk, we wanted to determine if GFP expression could be used
134 to facilitate the isolation of high-functioning single cell clones. We engineered the CRISPRa-sel

135 system into four unrelated cell lines and following puro selection we sorted cell populations based
136 on high, medium, or low GFP expression via fluorescence activated cell sorting (FACS) (Extended
137 Data Fig. 2c-d). From these sorted populations, single-cell clonal lines were derived, and upon
138 expansion were transduced with sgRNAs targeting distinct endogenous genes (PD-L1, CXCR4)
139 or a non-targeting control. Interestingly, while relative GFP expression levels were maintained in
140 the clones post-expansion (Extended Data Fig. 2c-left), there was no clear relationship between
141 reporter expression and endogenous target activation in three of four cell lines evaluated
142 (Extended Data Fig. 2c-center/right). These data suggest that, in the context of the CRISPRa-sel
143 system, selection with a CRISPRa dependent fluorescent reporter is not a broadly applicable
144 strategy for further enrichment of CRISPRa-competent populations, beyond what is achieved with
145 puromycin selection. While other groups have reported successful enrichment with fluorescent
146 CRISPRa responsive reporters²⁰, our data suggest such strategies are potentially more useful in
147 the context of low efficiency systems like the dual promoter or single transcript formats where the
148 number of active cells in the population is low and the functional difference between active and
149 inactive cells is high. On the other hand, GFP-based reporters may not be sensitive enough to
150 discriminate effectively between cells within more uniform CRISPRa-sel derived cell populations.
151 Therefore, we focused on antibiotic-selected populations for the remainder of our platform
152 optimization efforts.

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154

155 **SAM guide RNA scaffold optimization for enhanced CRISPRa activity**

156

157 Subtle changes in scaffold sequence and structure have been shown to affect guide RNA
158 function^{13,16,21,22} and we reasoned that the conventional SAM-2.0 scaffold could be re-engineered
159 to improve activity. The MPH activator utilized by the SAM system binds to two separate MS2
160 aptamers within the SAM-2.0 sgRNA; one in the tetraloop and one in stem loop two (Extended
161 Data Fig. 3a). Focusing on the tetraloop, we used rational design to create several new SAM-
162 compatible scaffold variants (Extended Data Fig. 3a-b, Fig. 2a). Previous reports have indicated
163 that Pol-III-based guide expression can be enhanced by removing a poly U tract in the tetraloop,
164 which can serve as a premature transcriptional termination sequence.^{21,22} (Extended Data Fig. 3b
165 [GNE-1]). Additionally, we hypothesized that increasing the stability or accessibility of the MS2
166 aptamer segment within the tetraloop could encourage greater associations with MPH complexes,
167 further improving CRISPRa efficiency. To explore these possibilities, we coupled poly U deletion
168 with an alternate, GC-rich stem extension sequence proximal to the MS2 aptamer (Extended Data
169 Fig. 3 [GNE-2])²¹. Finally, we combined both stem extension features with the removal of a bulge
170 sequence directly adjacent to the MS2 aptamer (Extended Data Fig. 3 [GNE-3]).

171

172 To evaluate the relative efficiency of these scaffolds, we lentivirally-transduced CRISPRa-
173 sel engineered K562 populations with sgRNAs targeting three endogenous genes (PD-L1, CD14,
174 or KDR) in either the SAM-2.0 scaffold format or one of our three novel variants (Extended Data
175 Fig. 3c). By flow cytometry, higher target expression was observed with several of the new
176 scaffold variants, but GNE-3 showed the most consistent improvement over 2.0, both in terms of
177 gene product levels (normalized MFI) and the percentage of activated cells across the population.
178 We subsequently expanded our comparison of the 2.0 and GNE-3 scaffolds to include six cell

179 surface receptor genes, using five unique sgRNAs per gene, to account for gene and spacer-
180 specific variability. Analysis of target transcript (qRT-PCR) and protein (flow cytometry)
181 expression (Fig. 2b-c; Extended Data Fig. 4a-b) revealed a broad enhancement of target
182 activation with the GNE-3 scaffold versus the 2.0 backbone, with several sequences achieving
183 between 5-10-fold improved gene induction with the GNE-3 variant. To confirm that the GNE-3
184 scaffold was beneficial in other cell contexts, we expanded our analysis to two additional cell lines.
185 As before, we found activation of PD-L1, as measured by cell surface staining in 293T and Jurkat
186 cells, (Extended Data Fig. 4c) was consistently higher with the GNE-3 scaffold. Taken together
187 these data suggest that the GNE-3 scaffold improves both the breadth and magnitude of gene
188 activation across a variety of spacer, target and cellular contexts. In addition, we found that
189 relative target gene activation was largely consistent when comparing transcript level or cell
190 surface protein stain (Extended Data Fig. 4d) for most targets, and therefore chose to move
191 forward with validated flow cytometry assays for subsequent experiments owing to the
192 quantitative nature of this assay at both the population and individual cell levels.

193

194 **CRISPRa-sel promoter optimization and evaluation in a panel of human cell lines**

195

196 While the combination of our CRISPRa-sel system with the GNE-3 scaffold demonstrated
197 improvement in overall CRISPRa efficiency, we continued to observe variable target activation
198 across cell lines (Fig. 3a-c [EF1 α], Extended Data Fig. 5 [EF1 α]). The strength of Pol-II promoters,
199 which drive expression of the CRISPRa machinery, can differ dramatically across cell types²³
200 potentially contributing to the context-dependent efficacy of CRISPRa (Fig. 3a). To evaluate how
201 promoter use impacts the efficiency of the CRISPRa-sel system, we engineered a panel of three
202 cell lines (K562, 293T and Jurkat) with the original EF1 α -based CRISPRa-sel vector or versions
203 that incorporated three distinct cytomegalovirus (CMV)-derived Pol-II promoter variants (CBh,
204 CMV, and CAG) (Fig. 3a-c.; Extended data 5) to drive expression of the activator machinery.

205

206 Attempts to engineer CRISPRa-sel populations were successful in all but one cell line
207 context (Jurkat + CMV-CRISPRa-sel) (Fig. 3b), in which only a low number of slow growing clones
208 were recovered following puromycin selection. To evaluate the relative efficacy of each promoter,
209 populations were transduced with GNE-3 sgRNAs targeting PD-L1 or CD2. Unlike the more
210 heterogeneous activation observed with the EF1 α , CBh, and CMV promoters, the CAG promoter
211 induced distinctly uniform and potent gene expression for each of the tested cell lines and targets
212 (Fig. 3b-c). We expanded our assessment to include three additional endogenous targets (CD14,
213 CXCR4 and CD69) and saw comparable results (Extended Data 5a-b). Importantly, this
214 demonstrated that population-wide CRISPRa was achievable with limited cell culture
215 manipulation steps beyond bulk antibiotic selection.

216

217 In order to confirm the broad utility of the CAG-CRISPRa-sel and GNE-3-sgRNA system,
218 we engineered an additional panel of ten commonly used cell lines (Fig. 3d-f). After bulk selection
219 of the CAG-CRISPRa-sel transgenic cell lines, introduction of a PD-L1-specific sgRNA led to
220 strong, uniform target induction (~79-99% of the cell population) (Fig. 3e-f). We then expanded
221 this analysis to four additional target genes per cell line, and while we observed some context-
222 dependent variability for individual genes, robust activation in $\geq 75\%$ of the cell population was

223 seen in the majority of conditions. Notably, beyond activating genes with little or no background
224 expression, we were able to induce population-wide upregulation of genes with high basal
225 expression (Fig. 3e [H358²⁴],[RKO²⁵]). Taken together these data indicate that the CAG-
226 CRISPRa-sel system in conjunction with the GNE-3 scaffold greatly enables the utility of stable
227 CRISPRa across a breadth of cell backgrounds and target genes.

228

229 **Optimized, multi-format synthetic guide RNAs for transient CRISPRa**

230

231 Synthetic guide RNAs can be generated quickly and have proven effective for Cas9-
232 mediated gene disruption purposes ranging from the creation of *in vitro* and *in vivo* models to
233 arrayed genetic screens²⁶. While synthetic gRNAs have previously been applied in the context
234 of CRISPRa²⁷ thus far they have not been widely adopted possibly due to their low efficiency with
235 sub-optimal CRISPRa systems. The production of synthetic, high-efficiency, SAM-compatible
236 guides has presented technical challenges. Until recently, dual MS2 aptamer-containing sgRNAs,
237 like the ~160 nucleotide GNE-3 spacer sequence and scaffold, exceeded the length of reliable
238 direct synthesis methodology²⁸. As an alternative approach, the use of easier-to-synthesize two-
239 part gRNAs (crRNA + tracrRNA scaffold) is an attractive possibility. The design of these guides,
240 however, must allow for efficient strand annealing while maintaining the structure of the MS2
241 aptamer loops²⁶. In addition, any synthetic guide RNA, regardless of format, needs to be stable
242 enough throughout the delivery, dCas9 association, and target binding processes to induce
243 measurable gene activation. Given recent advances in RNA synthesis and chemical stabilization,
244 and to yet further expand the utility of CRISPRa, we set out to develop an optimized GNE-3-based
245 synthetic gRNA platform.

246

247 To evaluate the impact of chemical modifications on the efficiency of CRISPRa induced
248 by transient delivery of synthetic guides in cultured cells, we synthesized a set of sgRNAs based
249 on the GNE-3 scaffold targeting four endogenous genes (PD-L1, CD14, CD2, CXCR4) with or
250 without modified stabilizing nucleotides²⁹. Individual unmodified sgRNAs were compared to
251 identical sgRNAs containing three terminal phosphorothioated 2' O-methyl ribonucleotides at
252 both the 5' and 3' ends (Extended Data Fig. 6a). Three days after electroporation into a CAG-
253 CRISPRa-sel-engineered K562 population, we observed clear evidence of gene activation. We
254 found that the modified sgRNAs demonstrated a clear advantage over the unmodified guides
255 across all targets evaluated (Extended Data Fig. 6b-c). To our surprise, activation with the
256 transient modified synthetic sgRNAs was qualitatively similar in some cases to stable sgRNA
257 expression, with near-population-wide expression achieved for two of four target genes.

258

259 We next sought to determine if the GNE-3 sgRNA variant also outperformed the 2.0
260 scaffold in a synthetic context. To this end we generated identical end-modified sgRNAs for the
261 2.0 variant. Direct comparison in the CAG-CRISPR-sel K562 model demonstrated a general trend
262 towards higher activation with the GNE-3 sgRNAs, although the differential was somewhat
263 reduced compared to the stable sgRNA context (Extended Data Fig. 7).

264

265 User accessibility of synthetic guide RNA-mediated CRISPRa could be enhanced by
266 lowering the cost and technical skill required for reagent synthesis. In principle, this could be

267 achieved by minimizing the length of the guide RNA segments with a more native, annealed two-
268 part crRNA-tracrRNA format. In order to create synthetic material that permitted crRNA and
269 tracrRNA hybridization while maintaining the GNE-3 scaffold loop structure, we developed two
270 distinct concepts (Extended Data Fig. 8a). In format 1, strand 1 includes the spacer sequence
271 and a segment of the GNE-3 MS2 containing tetraloop (Extended Data Fig. 8a-teal), which
272 anneals to strand 2 containing the final portion of the tetraloop as well as stemloop 1, stemloop 2
273 (with the second MS2 aptamer) and stemloop 3. Separately, in format 2, strand 1 exclusively
274 comprises the spacer plus a short region (Extended Data Fig. 8a- orange) with complementarity
275 to strand 2. Strand 2 of this format encodes the majority of the tetraloop and stemloops 1-3. All
276 RNA oligonucleotides contain 5' and 3' stabilizing modifications similar to our optimized synthetic
277 GNE-3 sgRNA. We incorporated identical spacer sequences within both formats and evaluated
278 their relative effectiveness for activating four separate genes within CAG-CRISPRa-sel K562
279 cells. When we analyzed target activation by flow cytometry three days post-electroporation we
280 saw higher gene activation with format 1 across all targets (Extended Data Fig. 8b-c), and this
281 format became a focus for follow-up studies.

282
283 Recently, a two-part, SAM-compatible guide RNA system has been described and made
284 commercially available²⁷. Unlike the GNE-3 guide RNAs described herein, the commercial
285 product contains fewer phosphorothioated 2' O-methyl ribonucleotides and has only a single MS2-
286 modified element within stemloop 2, the MS2 sequence within the tetraloop being notably absent
287 (Fig. 4a-top). In order to evaluate the relative functionality of these synthetic guide RNAs, we
288 compared GNE-3 sgRNAs and format 1 two-part guide RNAs to the commercially available
289 synthetic guide RNA format (1X MS2 two-part) in CAG-CRISPRa sel-engineered K562 and 293T
290 populations (Fig. 4 and Extended Data Fig. 9). We found that the GNE-3 sgRNA and two-part
291 formats generally outperformed the single MS2 containing guide (Fig. 4 and Extended Data Fig.
292 9) with the GNE-3 sgRNA format providing the most consistent and potent activation across all
293 tested contexts. The differential across guides was particularly pronounced in lower activity
294 conditions (Fig 4b-c, Extended Data Fig. 9a-b gRNA-1). Only under circumstances of high
295 CRISPRa activity, such as in 293T cells, could measurable induction be achieved with all of the
296 evaluated 2-part and sgRNA variants (Fig 4d-e, gRNA-3/gRNA-4, Extended Fig 9).

297
298
299 **Discussion**

300
301 The potential for any genome engineering technology is limited by the breadth of cell types
302 and loci for which it can be applied. By incorporating a unique self-selecting transgenic approach
303 with enhanced SAM-compatible guide RNA scaffolds, we have demonstrated that robust,
304 population-wide CRISPRa is achievable across a diverse panel of target genes and cell lines, all
305 with minimal cell manipulation steps. In addition, we show that synthetic guide RNAs can be
306 employed for highly-efficient, short term gene activation, in some cases with population-wide
307 efficacy. While this platform is expected to be broadly applicable, the required plasmid transfection
308 process may limit use in cell types that are sensitive to foreign DNA or difficult to transfect with
309 large plasmids. Adaptation of the self-selection concept with viral vector-based delivery could
310 circumvent this bottleneck.

311
312 Advancements in gene activator technologies are inevitable. With this in mind, we
313 anticipate that self-selecting circuits will be compatible with future transcriptional and epigenetic
314 modifier fusion proteins or extended Cas family member usage¹. This will be critical for expanding
315 the target space available for CRISPRa and for potentially enhancing gene expression at loci that
316 show weak or modest induction with the SAM activator machinery.

317
318
319 **Methods:**

320 321 **Cell culture, electroporation, transfection**

322 Cell line specific culture and manipulation protocols described in supplemental methods. All
323 parental cell lines were sourced from the Genentech cell bank (gCell) where they were maintained
324 under mycoplasma free conditions and authenticated by STR profiling. FACS sorting and
325 subsequent clonal derivation/analysis presented in extended data 2 c,d was performed by WuXi
326 AppTech.

327 328 **Lentiviral production/transduction**

329 sgRNA expressing and lentiviral packaging plasmids (VSVg/Delta8.9) were transiently co-
330 transfected into 293T cells with Lipofectamine 2000. Lentiviral supernatants were harvested at
331 72 hours and filtered through a 0.45 µm PES syringe filter (Millipore). Transduction with lentivirally
332 encoded guide RNAs performed as described in supplemental methods with cell line specific
333 protocols. 3 days following lentiviral infection, cells were started on zeocin selection at cell line
334 specific concentrations (supplemental methods) in order to select for guide RNA expressing cells.
335 Prior to gene expression analysis, uniform selection of gRNA infected populations was confirmed
336 by flow cytometric analysis of the co-expressed mTagBFP2 reporter.

337 338 **Flow cytometry**

339 Antibody staining performed using manufacturers recommended protocols and described in
340 supplemental methods. Data collection performed on BD FACS Celesta or BD FACS Symphony
341 machines and analyzed by FlowJo 2 10.8.0. Gating strategy indicated in Extended Data Fig. 1b.
342 Live cell populations were gated using FSC and SSC profiles. Where relevant, lentivirally
343 transduced cells specifically were examined by gating on mTagBFP2 positive populations. If cell
344 populations were selected to greater than >95% mTagBFP2 positive then this gating step was
345 omitted for some analyses. Populations were defined by gates established as indicated with 2
346 parameter pseudocolor plots (Extended Data Fig. 1b) with identical control cell lines expressing
347 a non-targeting control guide RNA and stained/collected in parallel.

348 349 **qRT-PCR**

350 RNA extraction performed with a Quick-RNA 96 well kit (Zymo). cDNA generation performed with
351 a high-capacity cDNA synthesis kit using random primers and RNase inhibitor (Thermo) following
352 recommended protocols. Quantitative RT-PCR performed with an ABI QuantStudio 7 Flex real
353 time PCR system. Relative quantification/fold change ($2^{-\Delta\Delta CT}$) analysis was performed by
354 QuantStudio software. A GAPDH control gene used for normalization purposes.

355 356 **Synthetic gRNA electroporation/transfection**

357 Direct synthesis and QC of the novel modified sgRNA and 2-part guide RNAs was performed by
358 IDT (<https://www.idtdna.com/pages>). All synthetic gRNAs were resuspended in Nuclease-Free
359 Duplex Buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate) (IDT). Commercially available

360 modified, synthetic 2-part guide RNAs containing a single MS2 aptamer loop purchased from
361 Horizon inc. (<https://horizondiscovery.com/>).

362
363 2-part crRNA and tracrRNA oligonucleotides were combined at equimolar ratios prior to a
364 denaturation/annealing protocol (95°C 5"; cool to room temp 2°/sec). sgRNAs were also treated
365 by heat denaturation prior to use. Cell line specific synthetic guide delivery protocols detailed in
366 supplemental methods.

367 **Data/Statistical analysis**

368 Statistical tests performed as indicated in figure legends for each experiment. Error bars represent
369 standard deviation from the mean. Data was analyzed using PRISM and/or excel software. Bar
370 plots/scatter plots and heatmaps were generated using PRISM.

372 **RNA structure prediction**

373 RNA folding performed using mFold³⁰ or bifold
374 (<https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/bifold/bifold.html>) algorithms.

376 **Figure production**

377 Figure elements produced in Excel (Microsoft), Flowjo (Becton Dickson) and PRISM (Graphpad
378 Software). Final figures created with BioRender.com.

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383 for thoughtful discussions and manuscript support.

385 **Disclaimers**

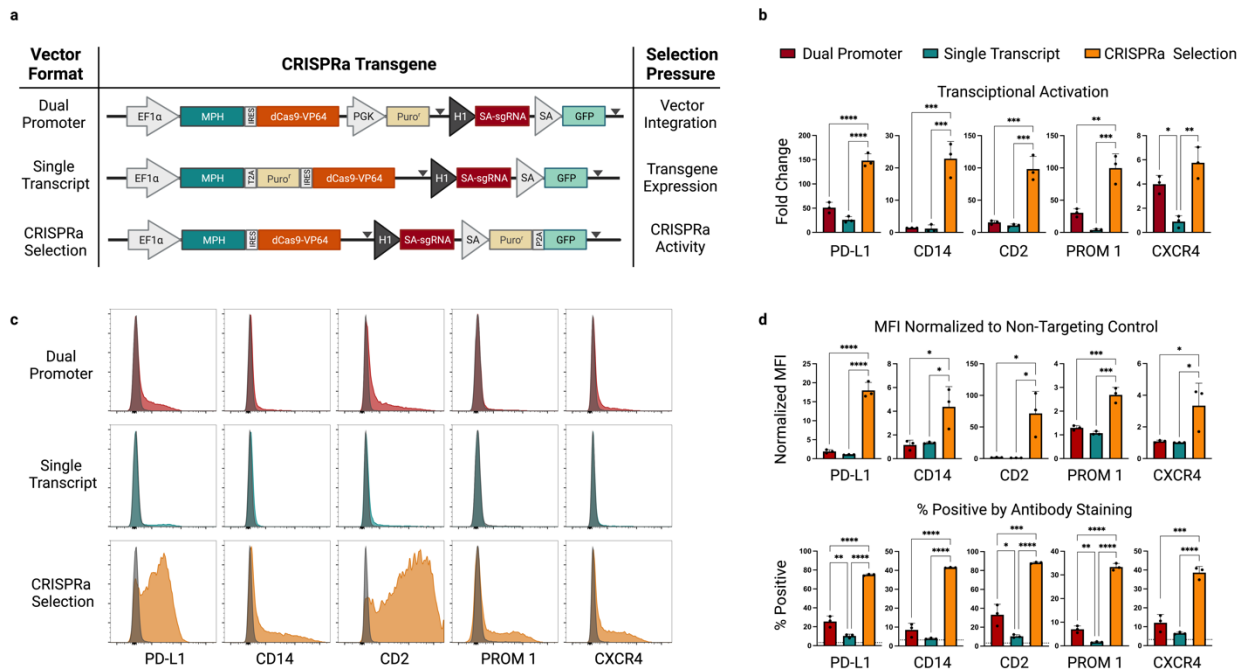
386 A.H., K.D., and B.H. are full time employees of Genentech, Inc. and shareholders of Roche.
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388 therapeutic purposes. Purchaser and/or user is solely responsible for all decisions regarding the
389 use of these products and any associated regulatory or legal obligations. J.A.G and A.M.J are
390 employees of Integrated DNA Technologies, which offers reagents for sale similar to some of the
391 compounds described in the manuscript.

393 **Data and Materials Availability Statement**

394 The datasets generated during and/or analyzed during the current study are available from the
395 corresponding author on reasonable request. Biological materials will be provided to requesters
396 through a material transfer agreement. Vector and guide RNA sequences are provided in
397 supplemental methods. Synthetic guide RNAs can be purchased through IDT.

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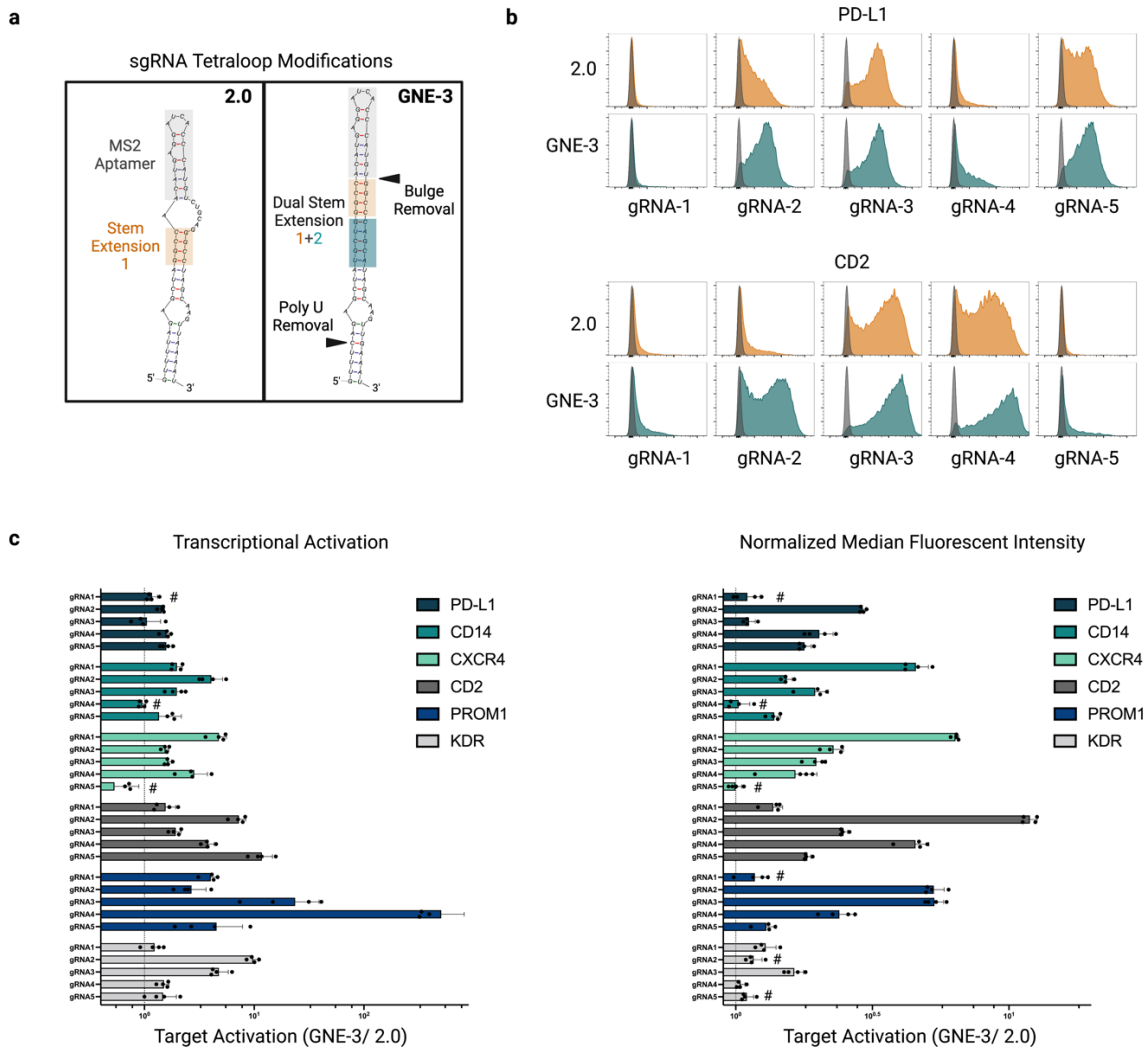
402 **Figures and Figure Legends:**
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Fig. 1: A self-selecting CRISPRa piggyBac vector for the rapid generation of stable, high-efficiency CRISPRa cell populations.

409 **a**, Vector format and selective strategy for the evaluated piggyBac CRISPRa expression-reporter vectors.
410 Expression of the MCP-P65-HSF1 (MPH) activator and dCas9-VP64 is driven by a constitutive, human
411 EF1α promoter. A human H1 promoter drives constitutive expression of a sgRNA complementary to the
412 self-activating (SA) promoter upstream of a GFP reporter. Expression of a puro^r gene is driven either by its
413 own constitutive promoter (dual promoter), transcriptionally linked to the MPH/ dCas9-VP64 (single
414 transcript) or under control of the CRISPRa dependent SA promoter (CRISPRa selection). Grey triangles
415 indicate the location of LoxP sites. PiggyBac engineered K562 populations were generated in triplicate for
416 each vector format and enriched with puro selection. sgRNAs complementary to the promoter proximal
417 region of the indicated genes were cloned into a lentiviral vector context containing a mTagBFP2/zeocin
418 selection cassette (Extended data 1a). Following transduction and zeocin selection target gene expression
419 was evaluated by quantitative RT-PCR (qRT-PCR) **b**, and flow cytometry at day 14 post-infection (**c-d**).
420 Representative histograms for each condition are overlaid with histograms from stained cell populations
421 expressing a non-targeting control gRNA (**c**) (gray). Infections were performed in duplicate and averaged.
422 (Median fluorescence intensity (MFI) was normalized to MFI of an antibody-stained sample expressing a
423 non-targeting gRNA (**d, top**). Percentage of cells positive by antibody staining is presented (**d, bottom**)
424 and background staining from a control sample expressing a non-targeting gRNA is indicated with a dashed
425 horizontal line for each gene. Statistical comparison was performed by an unpaired 1-way ANOVA. * p<0.5,
426 ** p<0.01, *** p<0.001. EF1α-Elongation factor alpha, GFP-green fluorescent protein, dCas9-vp64-
427 nuclease dead spCas9+vp64 activator fusion, P2A-porcine teschovirus-1 2A self-cleaving peptide, HSF-
428 heat shock factor, PD-L1-Programmed death-ligand1 (CD274), CD14-cluster of differentiation 14, CD2-
429 Cluster of differentiation 2, Prom1-prominin-1 (CD133), CXCR4-C-X-C chemokine receptor type 4
430 (CD184).
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434 **Fig. 2: Relative CRISPR activation efficiency of sgRNAs containing an optimized MS2 aptamer**
435 **containing scaffold.**

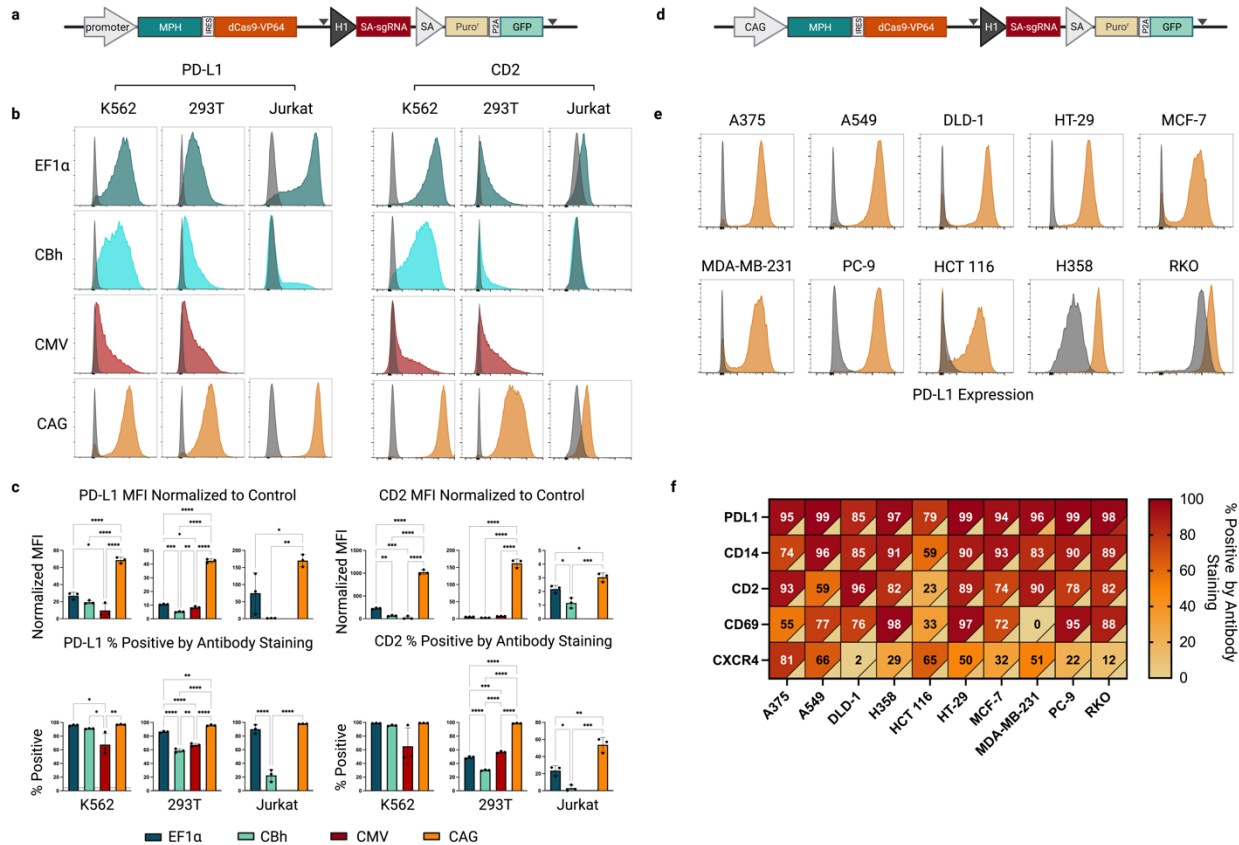
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437 **a**, Structure diagram of the MS2-aptamer containing tetraloop in the 2.0 sgRNA format¹³ (left) or an
438 optimized tetraloop structure (right and Extended Data Fig. 3). The optimized GNE-3 tetraloop contains an
439 additional stem extension and removal of a polyU tract²¹. Additionally, the bulge region connecting the MS2
440 aptamer and stem extension region 1 in the 2.0 format has been removed. **b**, Flow cytometric analysis of
441 target gene activation by sgRNAs with either a 2.0 (orange) or GNE-3 (teal) scaffold context. Representative
442 histograms of analyzed K562 CRISPRa-sel populations infected with 5 distinct spacer sequences targeting
443 the promoter proximal region of PD-L1 (top) or CD2 (bottom). Populations infected with a non-targeting
444 sgRNA sequence overlaid (gray). **c**, Activation of 6 target genes by GNE-3 sgRNAs normalized to the
445 activation efficiency of the same spacer sequence in a 2.0 format (dashed line). Normalized gene activation
446 was evaluated in zeocin selected populations by qRT-PCR (left) at day 14 post-sgRNA infection or by flow
447 cytometry (right) at day 10 post-sgRNA infection. n=4 replicates per sgRNA.

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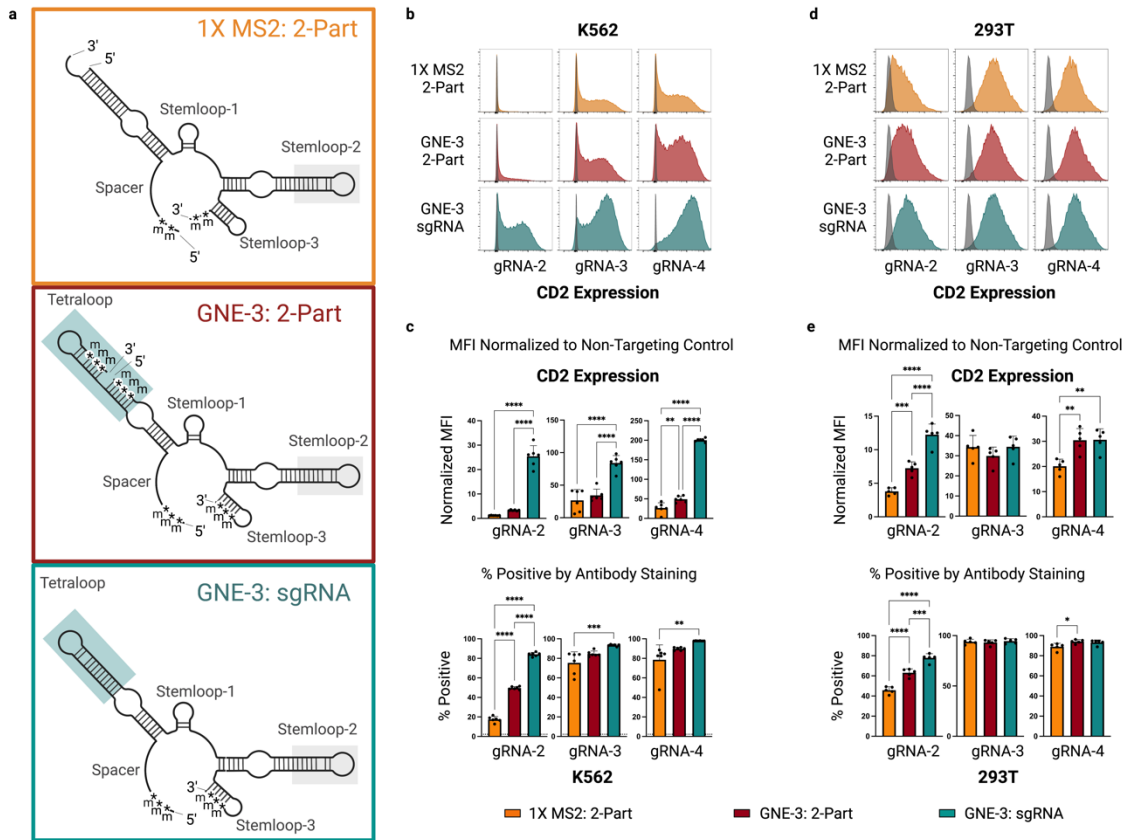


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453 **Fig. 3: Promoter optimization and application of the CRISPRa-sel strategy across a panel of commonly**
454 **used cell lines.**

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456 **a**, Schematic representation of the CRISPRa-sel vector indicating the location of the promoter driving
457 expression of the MPH/dCas9-VP64 transcript. **b-c**, Activation of PD-L1 (left) or CD2 (right) target genes
458 evaluated by flow cytometry in K562, 293T and Jurkat cell lines engineered with CRISPRa-sel piggyBac
459 vectors utilizing an EF1 α (teal), CBh (aqua), CMV (maroon) or CAG (orange) promoter 14 days post-infection
460 with a GNE-3 sgRNA. **(b)** Activation displayed by representative histograms overlaid with expression profiles
461 from cells infected with a non-targeting sgRNA (gray). **(c)** Normalized median fluorescence intensity (MFI) (top)
462 or percentage positive (bottom) of indicated genes/cell populations by antibody staining. The percent positive of
463 stained control populations infected with a non-targeting sgRNA are indicated by a dashed horizontal line.
464 (Note: *CMV CRISPRa-sel Jurkat populations did not grow out efficiently and were not included in the*
465 *analysis.*) **(d)** Schematic representation of the CAG CRISPRa-sel piggyBac vector. **(e)** Representative flow
466 cytometric histograms of PD-L1 activation across 10 commonly used cell lines engineered with a CAG-driven
467 CRISPRa-sel piggyBac vector and PD-L1 targeting GNE-3 sgRNA. **(f)** Heatmap representing the percent
468 positive of 5 target genes (PD-L1, CD14, CD2, CD69 and CXCR4) across 10 CAG CRISPRa-sel engineered
469 cell lines (A375, A549, DLD-1, H358, HCT 116, HT-29, MCF-7, MDA-MB-231, PC-9 or RKO). Percent positive
470 of stained cell populations expressing a non-targeting sgRNA represented colorimetrically in the lower right
471 corner of each cell. Gene-activating or control guides were expressed using dual sgRNA lentivectors
472 (supplemental methods). CRISPRa cell populations generated in triplicate and infected with indicated sgRNAs
473 in technical duplicates which were averaged before statistical comparison was performed by an unpaired 1-way
474 ANOVA. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$. Grey triangles indicate the location of LoxP sites. CBh -Chicken β -
475 actin hybrid promoter, CMV- human cytomegalovirus immediate-early gene enhancer/promoter or CAG- CMV
476 enhancer-chicken β -actin-rabbit β -globin synthetic hybrid promoter.

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480 **Fig. 4: Evaluation of CRISPRa synthetic guide formats across 2 cell lines.**

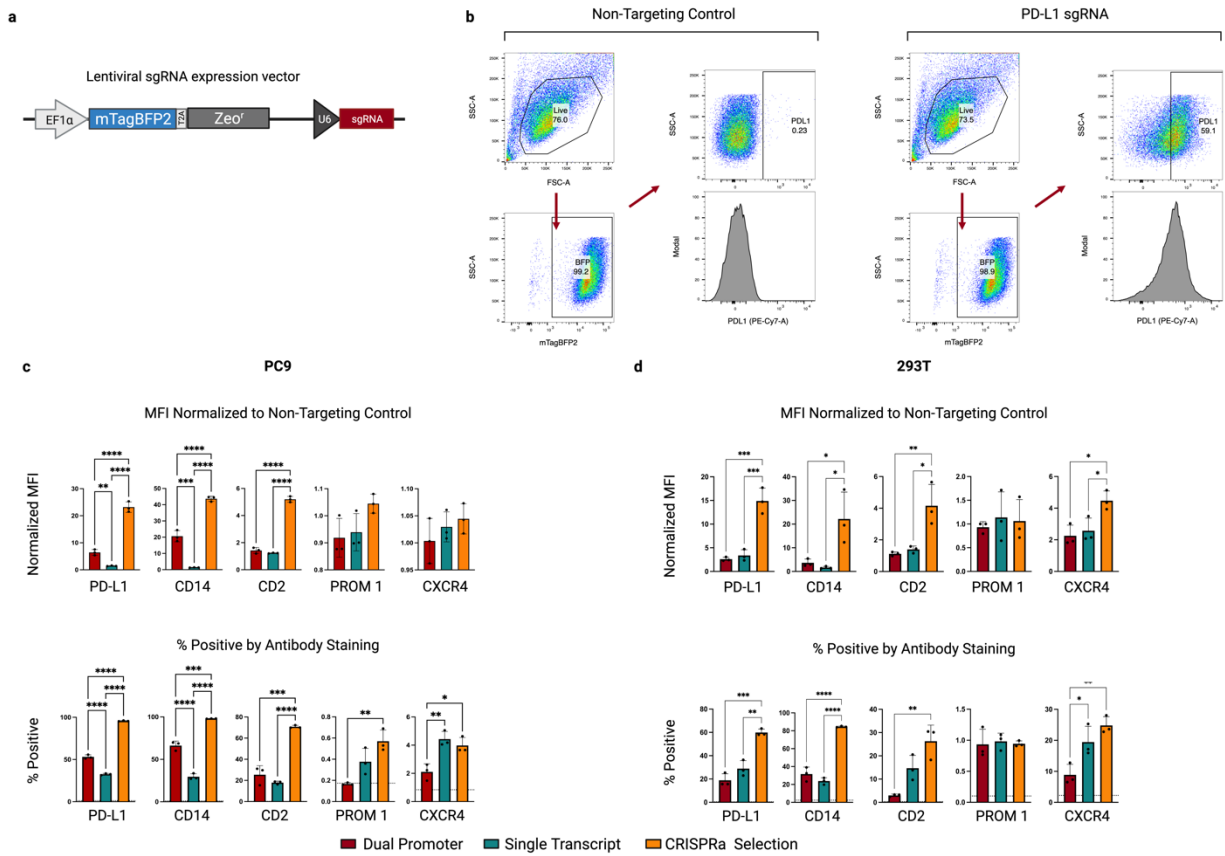
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482 **a**, Structure diagrams of a commercially available, chemically modified, 2-part synthetic gRNA containing
 483 a single MS2 aptamer loop (top-orange); a modified, 2-part format 1 synthetic gRNA containing a GNE-3
 484 scaffold (center-maroon); or a modified sgRNA with a GNE-3 scaffold (bottom-teal). Blue boxes highlight
 485 the MS2 aptamer-containing GNE-3 tetraloop and grey boxes indicate the MS2-aptamer on stemloop 2. **b-
 486 e** CRISPR-mediated transcriptional activation of a CD2 target gene in two CAG-CRISPRa-sel engineered
 487 cell lines (K562 or 293T) by electroporated modified, synthetic gRNAs in the formats depicted in **(a)**. CD2
 488 target expression by 3 spacer sequences in an engineered K562 cell line assessed by flow cytometry. CD2
 489 expression displayed by representative histograms overlaid with a control population **(b)** or summarized by
 490 median fluorescent intensity normalized to a non-targeting control **(c-upper)** or percent positive **(c-
 491 lower)**. Percent positive of a stained control population infected with a non-targeting sgRNA are indicated
 492 by a dashed horizontal line. **d-e**, CD2 target activation by synthetic gRNA formats as in b-c but in a 293T
 493 cell line. Flow cytometry performed 3 days after synthetic guide delivery. Statistical comparison between
 494 guide formats was performed by an unpaired 1-way ANOVA. * p<0.5, ** p<0.01, *** p<0.001. n=6 for K562,
 495 n=5 for 293T. m=2'-O methyl. *= phosphorothioate linker.

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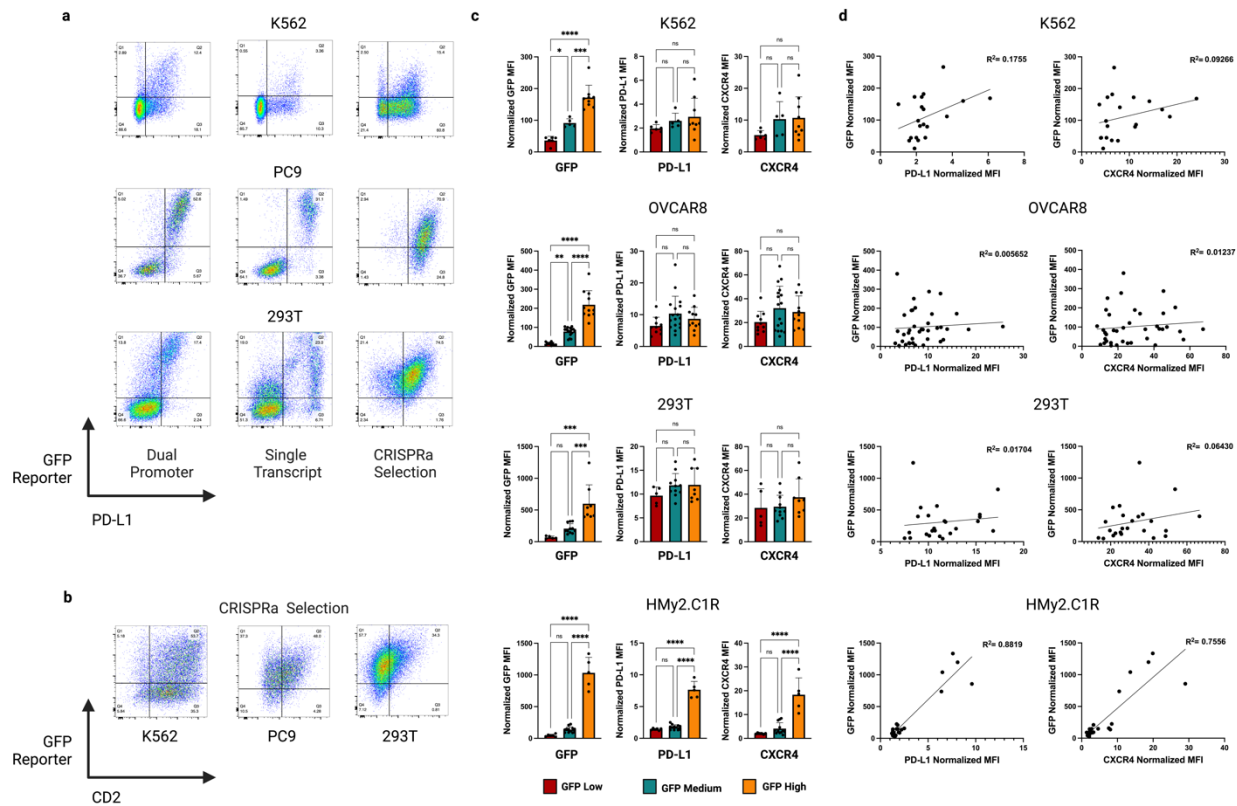
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Extended Data Fig. 1: Comparison of CRISPRa piggyBac vector systems in additional cell lines.

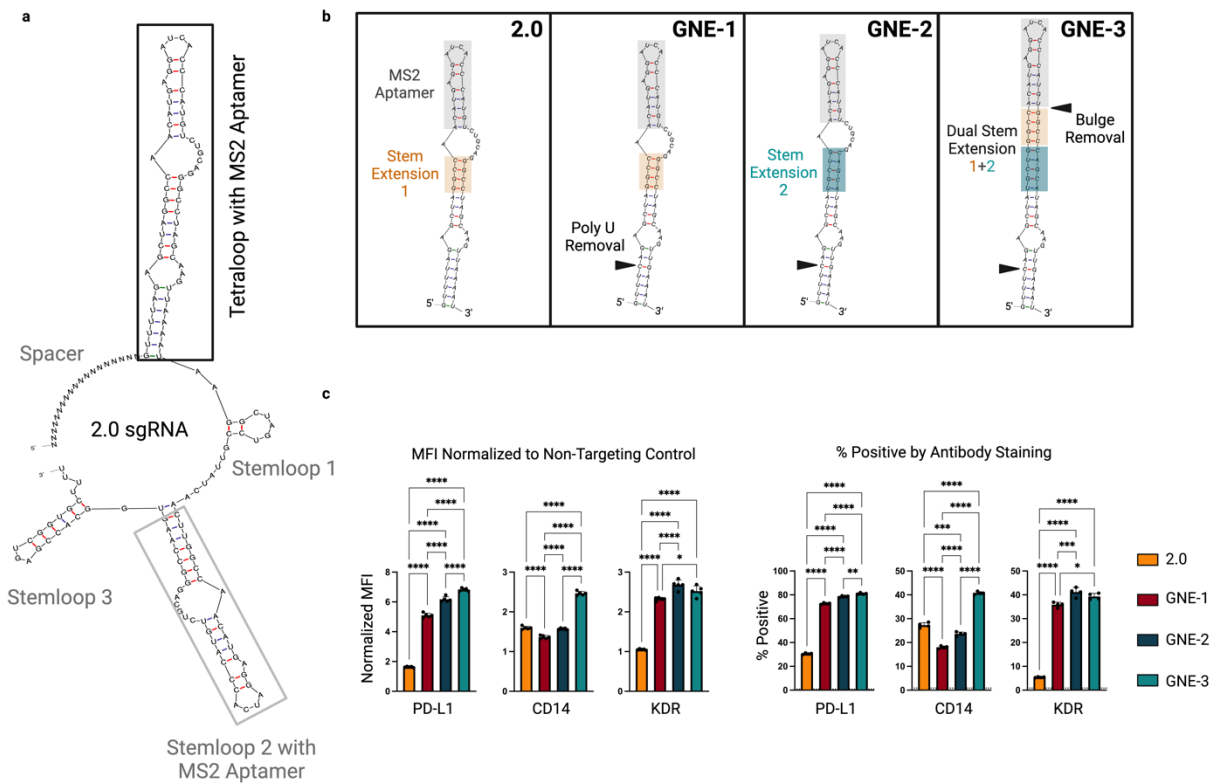
a, Schematic representation of the lentiviral sgRNA expression vector containing a mTagBFP2 marker and zeocin resistance (Zeo^r) selection cassette. **b**, Representative gating strategy for flow cytometric analysis shown in CRISPRa-sel populations expressing either a non-targeting control (left) or PD-L1 targeting sgRNA (right). Live populations were identified as indicated based on SSC-A (side scatter) and FSC-A (forward scatter) profiles and sgRNA expressing cells were identified by expression of the mTagBFP2 fluorescent protein. Positive population gates were defined in a control sample stained in parallel. **c**, Flow cytometric analysis of CRISPRa mediated gene expression in cell populations generated with three CRISPRa piggyBac systems utilizing distinct selection strategies (Fig.1). Analysis performed 14-25 days post lentiviral transduction of sgRNAs complementary to the promoter proximal regions of the indicated genes (PD-L1, CD14, CD2, PROM1 or CXCR4) for PC-9, or **d**, 293T cells. (Median fluorescence intensity (MFI) was normalized to MFI of an antibody-stained sample expressing a non-targeting gRNA (top). Percent antibody positive is presented (bottom) and background staining from a control sample expressing a non-targeting gRNA is indicated with a dashed horizontal line for each gene. Cell populations generated in triplicate. sgRNAs infected in duplicate and averaged prior to statistical comparison with an unpaired 1-way ANOVA. * p<0.5, ** p<0.01, *** p<0.001.



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Extended Data Fig. 2: An integrated CRISPRa dependent GFP reporter is an inconsistent marker of CRISPRa efficiency across multiple cell lines.

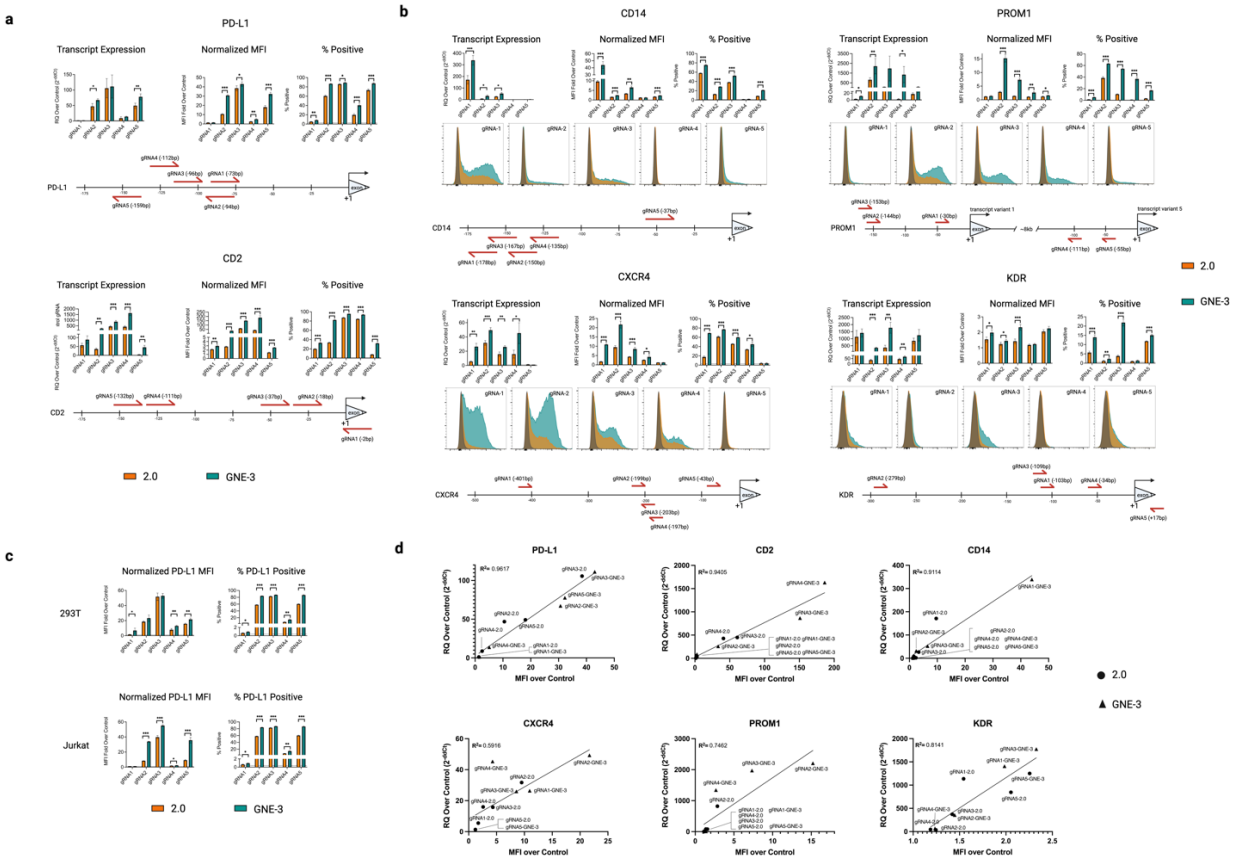
a, Flow cytometric analysis of GFP CRISPRa reporter vs endogenous PD-L1 target gene activation across three CRISPRa piggyBac formats in three cell lines (Fig. 1). **b**, GFP CRISPRa reporter vs endogenous CD2 activation in three cell lines engineered with a CRISPRa-sel piggyBac. **c**, Flow cytometric analysis of GFP CRISPRa reporter vs two endogenous CRISPRa target genes (PD-L1, CXCR4) in clones derived from CRISPRa-sel populations pre-sorted on GFP expression using flow assisted cell sorting (FACS) in four cell lines. Bar graphs of GFP median fluorescence intensity (MFI) in clones normalized to parental cell line (left). Target gene expression in engineered clones infected with an endogenous gene targeting sgRNAs (PD-L1 or CXCR4) and normalized to non-targeting control gRNA (middle/right). **d**, Scatter plots showing correlation of normalized MFI for CRISPRa dependent GFP reporter vs endogenous target gene activation. R squared for simple linear regression analysis indicated.



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542 **Extended Data Fig. 3: CRISPR activation efficiency of sgRNAs containing scaffold structural**
543 **modifications.**

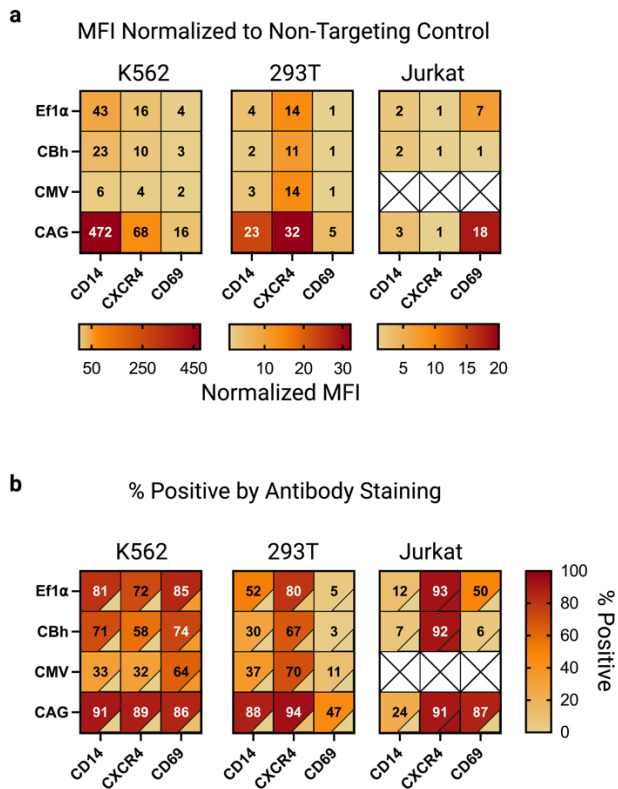
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545 **a**, Structure of the 2.0 sgRNA¹³ with a modified MS2 aptamer containing tetraloop (black box) and stemloop
546 2 (gray box). **b**, Enlargement of tetraloop structure with highlighted sequence modifications in the alternate
547 scaffolds evaluated. **c**, Flow cytometry data comparing activation efficiency of the four scaffold formats in
548 a K562 CRISPRa-sel population. Cell populations were lentivirally transduced with the sgRNAs targeting 3
549 endogenous gene targets (PD-L1, CD14 or KDR) and analyzed 7 days post infection. Data represented as
550 median fluorescence intensity (MFI) normalized to a cell population infected with a non-targeting sgRNA
551 (left) or percentage positive (right) with non-targeting gRNA represented by dashed horizontal line. n=4
552 technical replicates per condition. Statistical comparison was performed by an unpaired 1-way ANOVA. *
553 p<0.5, ** p<0.01, *** p<0.001. KDR-Kinase Insert Domain Receptor (VEGF2R/FLK1).
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Extended Data Fig. 4: sgRNA activation efficiency of guides in the 2.0 or GNE-3 scaffold context.

a,b, CRISPRa target gene activation by sgRNAs in a 2.0 (orange) or GNE-3 (teal) sequence context in a K562 CRISPRa-sel population. **(a)** Expression of PD-L1 (upper) or CD2 (lower) target genes assessed by qRT-PCR (left bar plots) and relative to a non-targeting control. Target gene expression assessed by flow cytometry and displayed by median fluorescence intensity (MFI) normalized to a non-targeting control (middle bar plot) or percent target positive (right bar plot). Background percent positive using a non-targeting control sgRNA indicated by horizontal dashed line. The position of guide RNA binding relative to the transcription start site (TSS) for each gene is indicated below. **(b)** Activation of 4 additional target genes with 2.0 or GNE-3 sgRNAs as assessed by transcript expression (top left), or flow cytometry (Normalized MFI- center; percent positive right or representative histograms, bottom panels). Guide position for each gene relative to the TSS indicated below. **c**, CRISPR mediated activation of PD-L1 in two additional cell lines (293T-top and Jurkat-bottom) by sgRNAs in a 2.0 or GNE-3 sequence context. PD-L1 expression assessed by flow cytometry and represented as MFI normalized to a non-targeting control (left) or percentage PD-L1 positive (right). Percent PD-L1 positive of cells infected with a non-targeting sgRNA represented by a horizontal dashed line. **d**, Scatter plots showing correlation of protein expression (normalized MFI) and transcript expression (qRT-PCR) for each sgRNA evaluated. R squared for simple linear regression analysis indicated. Statistical significance determined by a 2-tailed Student's t-test assuming unequal variance. * p<0.5, ** p<0.01, *** p<0.001. RQ-Relative Quantity



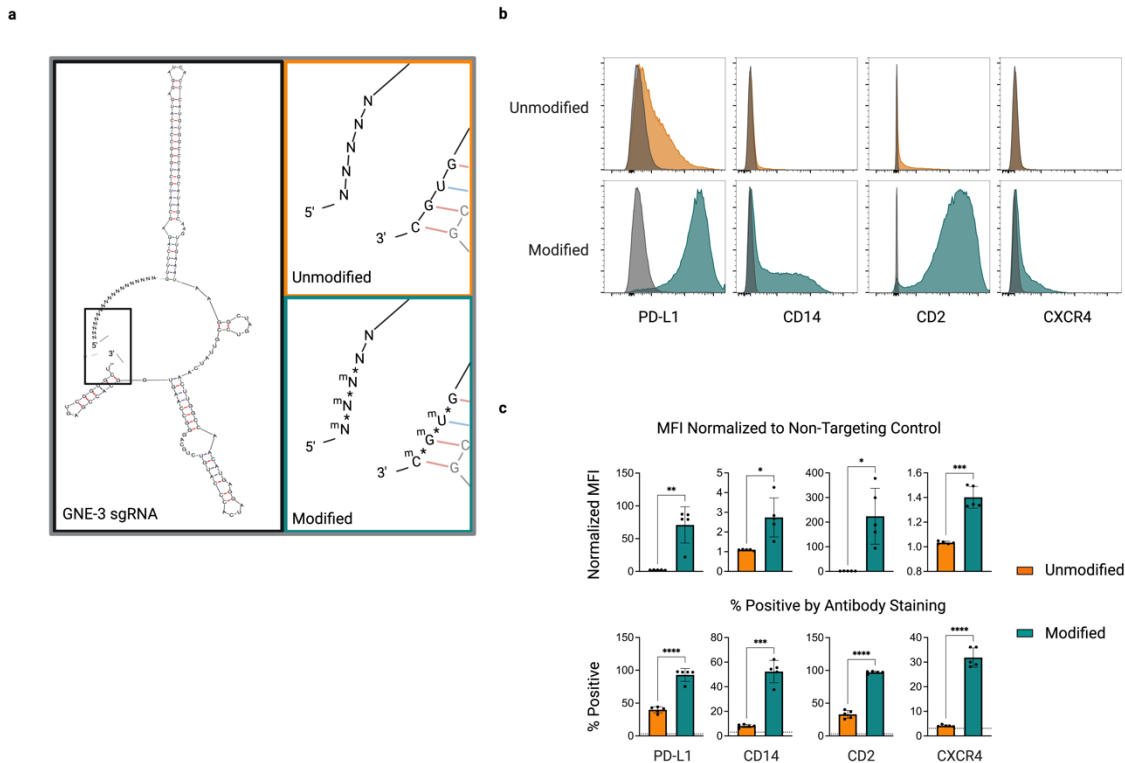
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Extended Data Fig. 5: Extended CRISPRa-sel promoter optimization and application in K562, 293T and Jurkat cell lines.

587 **a,b** Heatmap representing endogenous gene activation of 3 target genes in K562, 293T or Jurkat
588 populations engineered with CRISPRa-sel piggyBac vectors driven by the indicated promoters (Ef1 α , CBh,
589 CMV or CAG). Cells were infected with lentivirus encoding dual GNE-3 sgRNAs targeting the indicated
590 genes (CD14, CXCR4 or CD69) and assayed by flow cytometry 14 days post-infection/zeo selection. **(a)**
591 Median fluorescence intensity (MFI) normalized to a stained cell population infected with a non-targeting
592 control sgRNA in K562 (left), 293T (center) or Jurkat (right). Colorimetric scale for each cell line indicated.
593 **(b)** Percentage of the indicated cell populations positive by antibody staining. Percent positive of stained
594 cell populations expressing a non-targeting sgRNA represented colorimetrically in the lower right corner of
595 each cell. CRISPRa cell populations generated in triplicate and infected with indicated sgRNAs in technical
596 duplicates.

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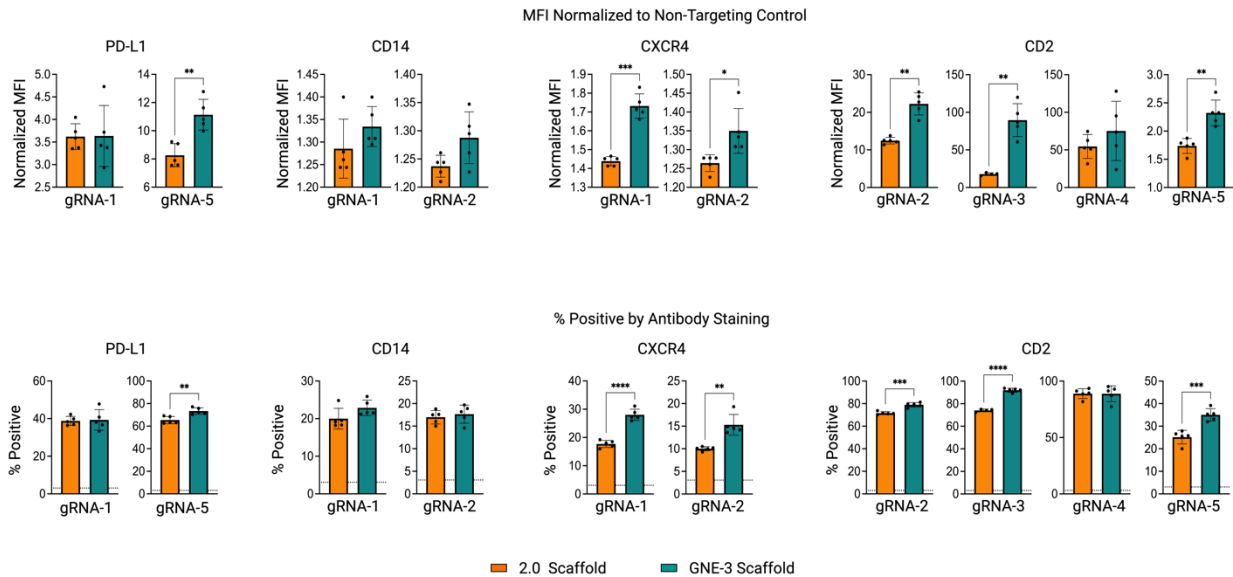
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Extended Data Fig. 6: Chemical modification of synthetic GNE-3 sgRNAs enhances target gene activation.

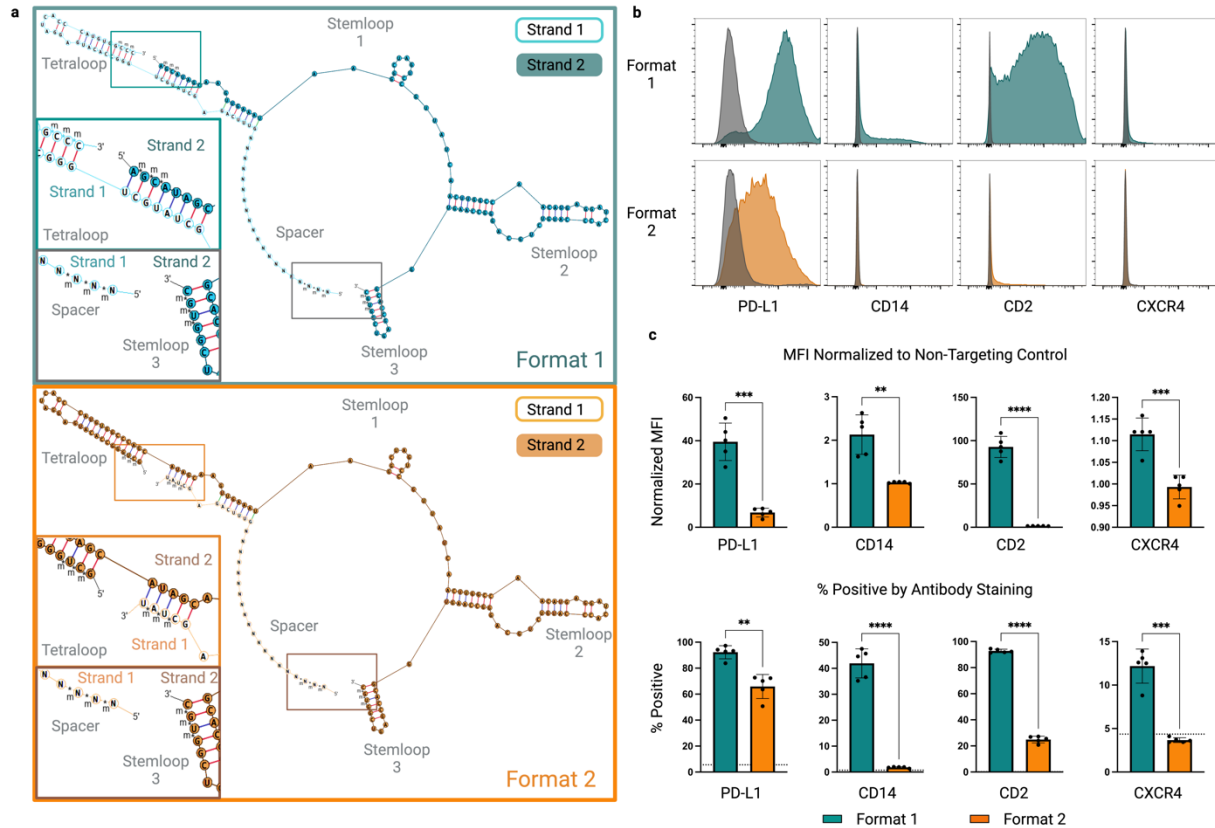
a, Structural diagram of a full sgRNA with GNE-3 scaffold highlighting 5'/3' ends (black box). Magnified view of sgRNA 5'/3' end regions highlighting unmodified (orange) or modified (teal) nucleotides. Modified sgRNAs contain 2'-O-methyl (m)/ phosphorothioate (*) linker modifications. **b,c** Assessment of CRISPR mediated gene activation by unmodified or modified sgRNAs in a CAG-CRISPRa-sel engineered K562 population and assessed 3 days post-sgRNA delivery. **(b)** Gene expression displayed by representative flow cytometry histograms in populations electroporated with unmodified (top row, orange) or modified (bottom row, teal) GNE-3 sgRNAs. Stained cells electroporated with a non-targeting synthetic sgRNA overlaid in gray. **(c)** Median fluorescence intensity (MFI) of K562 populations stained with antibodies for the indicated genes (PD-L1, CD14, CD2, CXCR4) and normalized to a population of stained cells electroporated with a non-targeting sgRNA (top). Percentage of cells positive by antibody staining (bottom). Background staining of a cell population electroporated with a non-targeting control sgRNA indicated with a dashed horizontal line. n=5 technical replicates per condition. Statistical significance determined by an unpaired 2-tailed t-test with a Welch's correction. * p<0.5, ** p<0.01, *** p<0.001.



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Extended Data Fig. 7: Activation efficiency of synthetic, modified, sgRNAs with a 2.0 or GNE-3 scaffold.

Evaluation of CRISPR mediated gene activation in a CAG-CRISPRa sel engineered K562 population electroporated with modified synthetic sgRNAs in a 2.0 (orange) or GNE-3 (teal) scaffold context. Activation of 4 target genes (PD-L1, CD14, CXCR4 or CD2) by flow cytometry 3 days post sgRNA electroporation. Data represented as normalized median fluorescent intensity (top) or percent positive observed by antibody staining (bottom). Background antibody staining indicated by horizontal dashed line. n=5 technical replicates per condition. Statistical significance determined by an unpaired 2-tailed, t-test with a Welch's correction. * p<0.5, ** p<0.01, *** p<0.001.

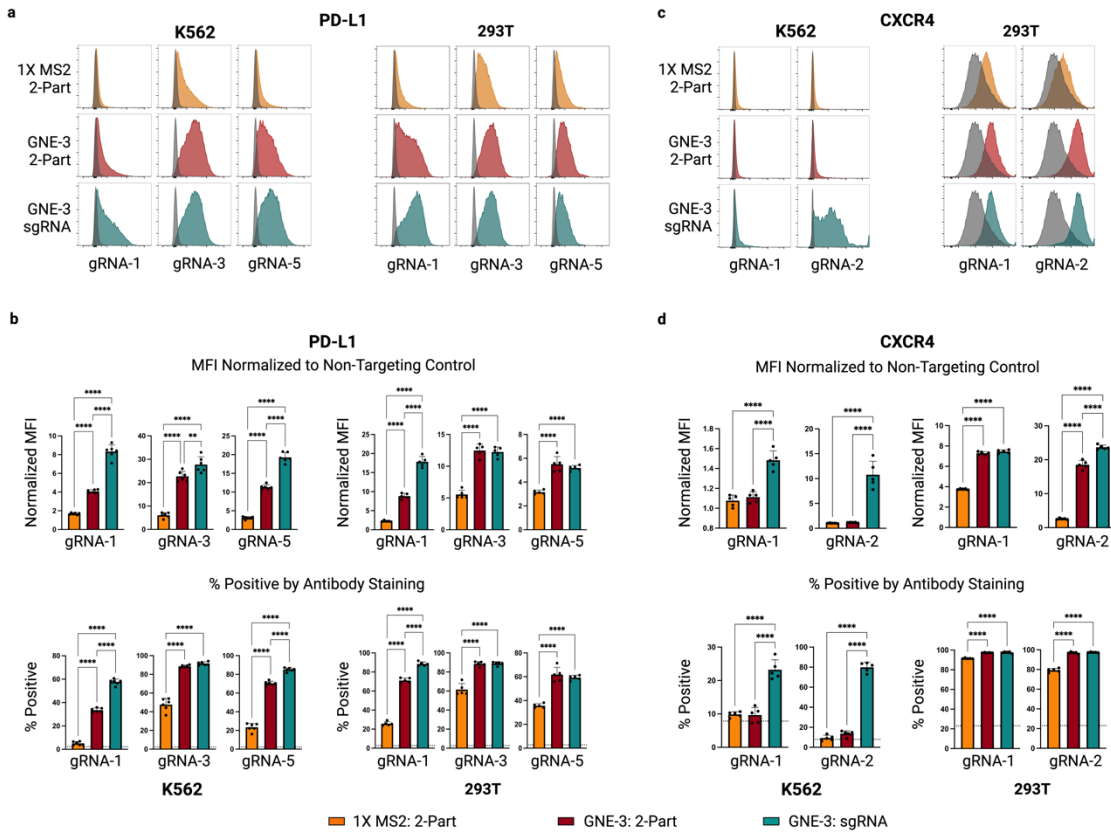


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Extended Data Fig. 8: Comparison of alternate synthetic, 2-part GNE-3 guide RNA formats.

a, Diagrams of alternate 2-part, modified, GNE-3 scaffold containing guide RNA structures. **Format 1** (teal) encodes a spacer and the majority of the MS2 containing tetraloop on strand 1. Strand 2 of this format encodes the remainder of the tetraloop as well as stemloop 1, stemloop 2 with a second MS2 aptamer and stemloop 3. Strand 1 of the alternate format 2 structure (orange) encodes a spacer sequence and a short 3' sequence predicted to anneal to the strand 2 scaffold containing the GNE-3 tetraloop and stemloops 1-3. Inset panels indicate the 2'-O-methyl and phosphorothioate linkers on the distal ends of each strand. **b-c**, Evaluation of alternate formats in CAG CRISPRa-sel engineered K562 populations and 3 days post gRNA electroporation. Expression of indicated endogenous target genes (PD-L1, CD14, CD2 or CXCR4) were evaluated by flow cytometry and presented as **b**, representative histograms overlaid with non-targeting control electroporated populations (gray) or **c**, summarized as normalized median fluorescence intensity (top) or percentage target gene positive (bottom). Background staining indicated by horizontal dashed line. n=5 technical replicates per condition. Statistical significance determined by an unpaired 2-tailed t-test with a Welch's correction. * p<0.5, ** p<0.01, *** p<0.001. m=2'-O methyl. *= phosphorothioate linker.

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658 Extended Data Fig. 9: Evaluation of CRISPRa synthetic guide formats in an expanded cell panel.

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660 **a-d**, Target gene activation comparing three different synthetic guide RNA formats, 1X MS2 2-Part (orange),
 661 GNE-3 2-Part (maroon) and GNE-3 sgRNA (teal) as illustrated in Fig. 4a, across CRISPRa-sel K562 and
 662 293T cell populations. **(a)** PD-L1 and **(c)** CXCR4 activation assessed by flow cytometry of antibody-stained
 663 cell populations 3 days post gRNA electroporation (K562) or transfection (293T). Representative
 664 histograms for each gene targeting gRNA are shown, overlaid with histograms for the non-targeting gRNA
 665 control (grey). Median fluorescence intensity for each gene targeting gRNA normalized to the non-targeting
 666 gRNA control for **(b, top)** PDL1 and **(d, top)** CXCR4. Percentage of cells positive by antibody staining for
 667 PD-L1 **(b, bottom)** or CXCR4 **(d, bottom)**, with background staining indicated by the horizontal dashed
 668 line. n=5 technical replicates per condition. Statistical comparison was performed by an unpaired 1-way
 669 ANOVA. ** p<0.01, **** p<0.0001.

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